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PRINCIPAL INVESTIGATOR: Martha Mims

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Martha Mims

E-Mail: mmims@bcm.edu

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14. ABSTRACT
We are examining the hypothesis that mitochondrial inheritance plays a significant role in aggressiveness of prostate cancer in African Americans. In the first year of the project we have identified 2,000 noncancerous tissues samples from African American men with prostate cancer and we have extracted DNA from ~ 1,500 of them to date. We have validated a robust new DNA sequencing technique developed by our collaborator using single amplicon long-range PCR that permits deep coverage (10,000-20,000X on average) of the mitochondrial genome. We have more than 300 samples in the sequencing pipeline with several hundred more to be submitted in the next few weeks. We have begun mapping of DNA variants in our sequenced genomes to mitochondrial genes. Illumina African Admixture mapping of the initial 72 subjects has been accomplished. Initial steps toward creating the Rho0 cell lines necessary for cybrid generation have begun with generation of puromycin resistant cell lines and testing of two different technologies for eliminating mitochondria from the nuclear donor cell lines.

15. SUBJECT TERMS
Mitochondrial DNA sequencing, Prostate cancer, Cybrid cells, African Americans.

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Introduction:

African American men are disproportionately affected by prostate cancer with increased lifetime risk, earlier onset of disease and more advanced stage at diagnosis than Caucasians. Prostate cancer is the leading cause of cancer death in African American men, with mortality more than double that observed in Caucasians. There are nearly 20 million African American men in the US, many of whom face significant risk of developing and dying from prostate cancer. Risk for and aggressiveness of prostate cancer in African American men is thought to originate in part from genetic susceptibility. Several nuclear genes and chromosomal regions have been linked to prostate cancer; however, many studies have not included African American men, and no study has linked genetic polymorphisms with clinical outcome. One factor which has not been carefully examined is mitochondrial inheritance which varies significantly between ethnic and racial groups and could explain large differences in disease characteristics. In the present study, we are examining the hypothesis that mitochondrial inheritance plays a significant role in aggressiveness of prostate cancer in African Americans. Further, we predict that adverse clinical outcomes will be reflected in dysregulation of cellular biochemical processes and in alterations in signaling pathways (Akt pathway, apoptosis). The ability to identify mitochondrial variants or haplogroups that contribute to aggressive disease will help to separate patients with indolent disease who can be spared unnecessary intervention from those who need more immediate and aggressive therapy. Identification of cellular pathways involved will help to target treatment strategies for those with cancers predicted to be more aggressive. In Aim 1 we will conduct a study of mitochondrial inheritance in 1,000 African American men with prostate cancer. We will sequence the mitochondrial genome of all 1,000 samples and determine whether particular mitochondrial variants, genes or haplogroups are associated with markers of aggressive disease (age at diagnosis, stage at diagnosis (including bone metastasis), Gleason score, PSA at diagnosis, PSA recurrence and death from disease). Findings will be replicated using an independent set of 1,000 patients from our own tissue resource with linked clinical data. Our strategy will be to sequence the mitochondrial DNA of all 1,000 patients in the discovery phase as well as in the replication group. We will control for population admixture using the Illumina African American Admixture Panel. Using cybrid technology, we will introduce our previously identified mt10398A variant and mitochondrial variants associated with highly aggressive and least aggressive disease identified in our genotyping study into prostate cell lines (derived from normal and cancer cells). Cybrids differing only in their mitochondrial composition will be examined for viability under metabolic stress, cell cycle distribution, production of reactive oxygen species, O_2 consumption, ATP synthesis, respiratory chain activity and capacity to grow in an anchorage independent manner. The effect of mitochondrial variants on nuclear gene expression will be studied using Western blotting and microarrays.

Body:

In the text to follow, we provide the Aims and original Statement of Work in italics with progress on the project presented in regular text.

<u>Aim 1:</u> To examine the association between mitochondrial DNA variants and clinical outcome in African American men with *prostate cancer.*

Task 1: <u>Extraction of all DNA for Initial Study and Validation Set</u>

1a. Complete extraction of DNA/ quantitation of DNA for mitochondrial sequencing *Months 1-9*
(Extraction ongoing at present, expect more than 1,000 samples to be prepared before the project is funded. Additional 1,000 to be extracted for validation set IRB protocol already approved)

To date we have extracted DNA on more than 1500 Tissue samples from African American Men with Prostate cancer. Work has been a bit slower than we had initially planned because of difficulties with extracting adequate DNA from some of the FFPE (paraffin-embedded) samples. We have consulted with colleagues at the University of Arizona for additional insight. Recommendations have been: 1) to extract only 2-3 10 micron sections per tube with 2 tubes per sample to make extraction more effective and increase DNA yield; 2) to allow the ethanol to dry completely off the deparaffin preparation prior to lysis to increase the effectiveness of the lysis; 30 to heat at 90^0C for 2 hours which reducs cross-linking and allows more efficient nucleic acid extraction

especially for older specimens. With these modifications we are increasing our yields on smaller volume and older samples.

Task 2: <u>Mitochondrial Sequencing</u>

2a. Order and test overlapping mitochondrial primers with FFPE DNA to be sure that all primers work with FFPE tissue DNA	*Ongoing – Month 3*
2b. Redesign/test and mitochondrial primers which do not give good PCR results in 2a.	*Months 4-6*
2c. Establish Database for mitochondrial sequences	*Months 1-5*
2d. PCR mitochondrial sequences for primary study and validation set	*Months 6-20*
2e. Sequence mitochondrial PCR products	*Months 6-20*
(Illumina Admixture Genotyping to be supported by Helis funds, but performed simultaneously)	*Months 6-20*

As reported in our initial project proposal, based on published sequences for mitochondrial PCR primers[1, 2] we designed 61 pairs of overlapping primers to amplify the entire 16.6-kb mitochondrial genome. In order to test this technology, we used 15 matched DNA samples derived from 5 patients – frozen tissue, FFPE tissue and whole genome amplified DNA samples derived from FFPE DNA for each patient. Overall 2/3 of the amplicons had reads in both directions on all 15 samples (including WGA DNA). Five of 61 amplicons failed completely and will be redesigned. Thus, we covered 93% of the genome by reads in at least one direction in 90% of the samples, and by reads in both directions in 84% of the mt genome, an excellent outcome for a first pass analysis over a target. Using these results, we identified 165 variants as compared to the published mitochondrial sequence. Within these variants there was 96-100% concordance of calls for each patient across the three sample types. We have continued to optimize the primers for the Sanger sequencing protocols, but as we reported in our initial application, we have also continued to test new technologies for mitochondrial sequencing and we are pleased to report here that we have obtained excellent results using an new technique developed by one of the collaborators on the this project (Dr. Lee-Jun Wong). This technique enriches the entire human mitochondrial genome by a single amplicon long-range PCR followed by massively parallel sequencing.[3] This protocol, described briefly below utilizes less than 100 ng of tissue DNA and makes possible a one- step approach to provide quantitative base calls, exact deletion junction sequences and quantification of deletion heteroplasmy.

As described in more detail in Zhang et al[3], the forward and reverse primers were: mt16426F (5′-CCGCACAAGAGTGCTACTCTCCTC-3′) and mt16425R (5′-GATATTGATTTCACGGAGGATGGTG-3′). PCR is performed using a TaKaRa LA *Taq* Hot Start polymerase kit (TaKaRa) and 50 ng of total genome DNA isolated from frozen seminal vesicle as template in a 50-μL PCR system. An initial 2-min incubation at 95 °C was followed by 30 cycles of PCR with 20 s of denaturation at 95 °C and 18 min of annealing and extension at 68 °C. The reaction was completed by 1 cycle of final extension at 68 °C for 20 min. Indexed paired-end DNA libraries were prepared according to the manufacturer instructions with minor modifications. Briefly, LR-PCR products were fragmented to 200 bp, which were purified with AMPure XP beads. After end repair, 3′-adenylation, and Illumina InPE adapter ligation, DNA samples were enriched by PCR with Herculase II polymerase (Agilent Technologies). Twelve indexed DNA libraries were pooled together with equal molar ratios. Each pooled library was sequenced in a single lane of 1 flow cell on HiSeq2000 with a 76- or 100-bp paired-end or single-end read chemistry.

Figure 1 shows the results from the first few samples sequenced by this new technology. We now have 300 samples in the sequencing pipeline with an additional 200 to follow in the next few weeks. As seen in figure 1, a distinct advantage provided by amplification of the entire mitochondrial genome by long range PR with a single primer pair is the uniform coverage. This figure demonstrates that we routinely had coverage depth of 10,000-20,000-fold for the mitochondrial genome. This sequencing strategy has multiple advantages including ease of excluding nuclear DNA sequences which are nearly identical to mtDNA and more uniform coverage of

the mitochondrial genome because there is much less risk of having a rare or novel variant at the primer binding site when only one set of primers is used. Deep coverage allows much more accurate base calls and improves the limit of detection for heteroplasmy from about 15% with Sanger sequencing to ~ 1-2% with the long range primer technique. Though we are not certain how well this technology will work on the FFPE samples, we intend to take full advantage with frozen tissue samples to rapidly obtain and process mtDNA sequences.

Figure 1: Sequence coverage for 4 representative DNA samples across the entire mitochondrial genome

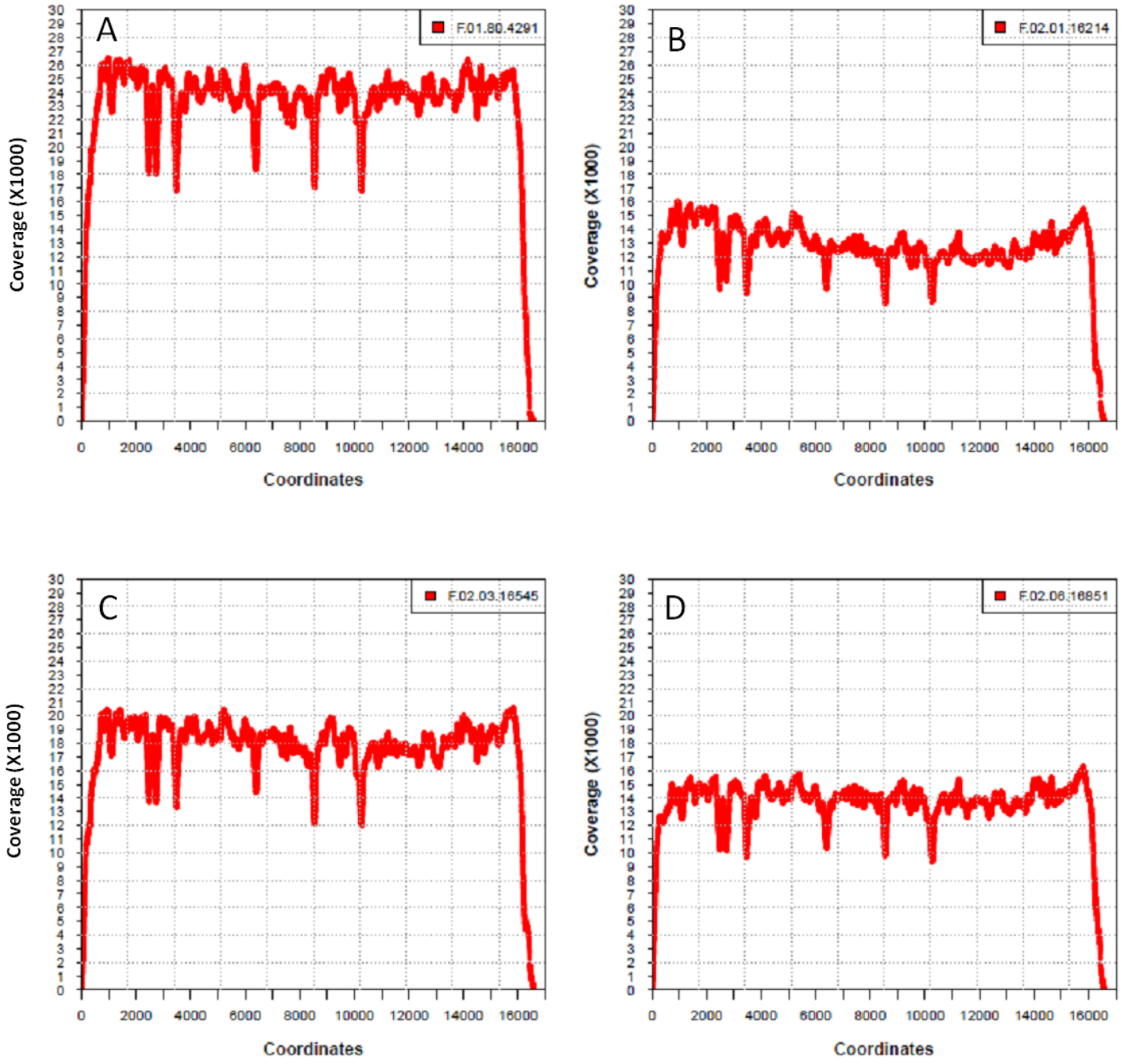

Figure 1: Panels A-D show representative coverage for 4 different DNA samples across the entire 16,569 bp mitochondrial genome. The y axis represents fold coverage at each position (x coordinate) along the mitochondrial genome

Figure 2 demonstrates the variant ratios for the same 4 representative sequences shown in figure 1. The Cambridge mitochondrial sequence is used as reference.[4,5] Note that unlike nuclear DNA where heterozygosity is possible, most data clusters near 0 or 1. Since the mitochondrial genome is essentially haploid this suggests there there is little contamination with nuclear DNA sequences.

Figure 2: Variant ratios at each position along the mitochondrial genome for 4 representative DNA samples

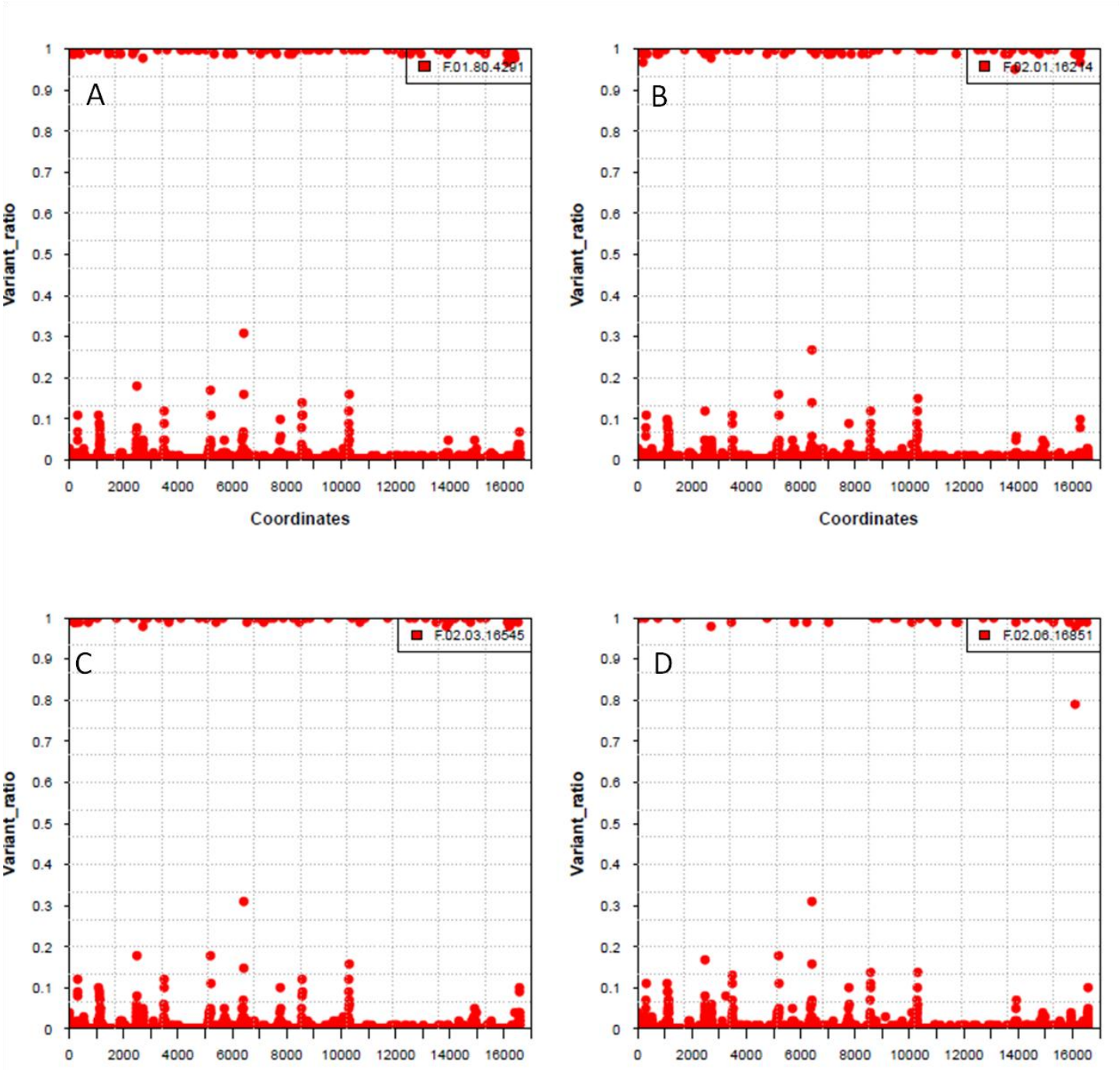

Figure 2: Panels A-D variant ratios for 4 different DNA samples across the entire 16,569 bp mitochondrial genome. Variant ratio is calculated as number of variant reads (as compared to the Cambridge Sequence) divided by total number of reads at each position.

We have now created a database of the sequence data which will ultimately include not just mtDNA sequence data, but nuclear SNPs gleaned from the Illumina Race/Ethnicity panel (see below). For purposes of demonstration, we have mapped the first 8 sequences obtained to the mitochondrial genome (see figure 3 below) to demonstrate the distributions of the mutations found in the various mitochondrial genes. Mutations below the 0.01 variant ratio have been filtered out.

Figure 3: Distribution of variants found in mitochondrial genes for 8 representative subjects.

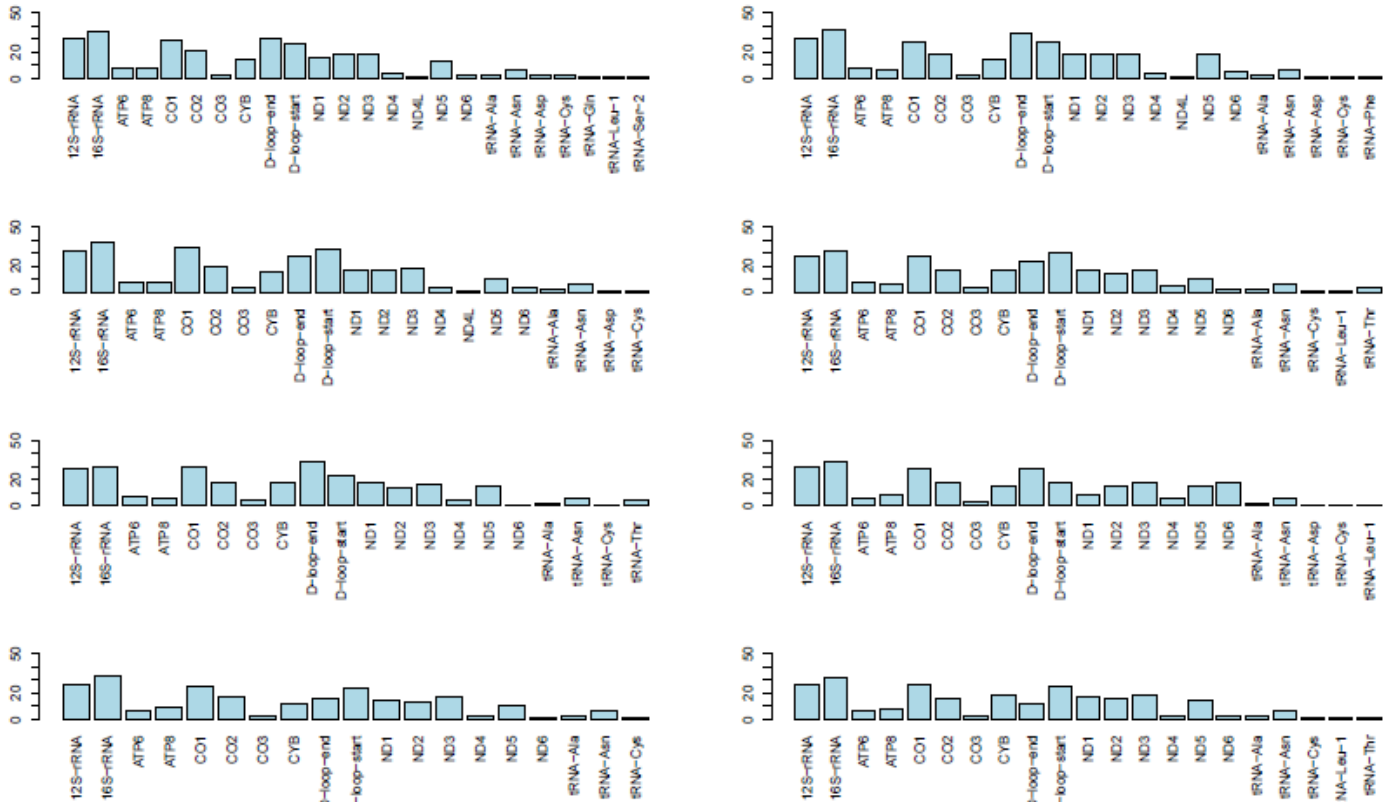

Figure 3: Each panel represents the distribution of mitochondrial variants found within various mitochondrial genes for 8 individual subjects as compared to the Cambridge reference sequence. The Y axis represents the absolute number of variants observed within each gene.

We are hopeful that this new sequencing methodology will supplant the need to conduct Sanger Sequencing with overlapping primers; however, we are not certain that the technology will be as successful with the FFPE DNA, thus we are continuing to work on optimizing the primer pairs should that be necessary to proceed with the FFPE DNA samples.

Although not covered by this project, we have also sent the first 72 samples for genotyping on the Illumina African American Admixture panel. All 72 samples were successfully genotyped.

<u>Aim 2:</u> To understand the role of mitochondrial DNA variants in regulation of cellular processes important in the cancer cell including those involved with generation of reactive oxygen species and energy metabolism.

Task 1: <u>Creation of Cybrid cell lines</u>

1a. IRB submission for creation of lymphoblastoid lines from patients	*Months 1-4*
1b. Creation of immortalized lymphocyte bank for use in study	*Months 5-24*
1c. Order/test primers for variable number tandem repeat studies of nuclear origin	*Months 6-12*

1d. Deplete prostate cancer cells of mitochondria *Months 9-24*
3 potential cell lines to be used: PNT1A, LNCaP or PC3

In collaboration with Dr. Michael Ittmann, the IRB submission for creation of lymphoblastoid cell lines was obtained before the project was actually funded. We have not yet begun to create the cell lines because we are unsure of which patient cells we will need to conduct our experiments.

We have begun to make mitochondria-depleted Rho^0 cells from prostate cancer cell lines PC3, LNCaP and PNT1A. These cells were treated with Ethidium Bromide (EB) at varying concentrations. To confirm mitochondrial DNA (mtDNA) depletion, the mtDNA content was intermittently checked by qPCR analysis. Initially, among the three cell lines, the mtDNA copy number decreased significantly in benign PNT1A cells as compared to the PC3 and LNCaP. The dosage of EB for the cancer cells PC3 and LNCaP was increased to varying concentrations. Though the mtDNA content in PNT1A cell lines decreased considerably, a mtDNA persisted without the support of external uridine. Since this condition is not enough for the cybrid generation, the EB treatment has been continued for all the cells at varying concentrations. We are checking mtDNA content qPCR to evaluate the progress of mtDNA depletion.

Since mitochondrial DNA depletion by the EB method requires more time and produces variable results depending on the cell model, we are utilizing in parallel Rhodamine6G (R6G) treatment for cybrid generation. The prostate cell lines were treated with different concentrations of R6G ranging from 3 μg/ml to 1μg/ml for a short period of time. We expect gradual death of cells after R6G treatment if the cells are not fused with mitochondria from donor cells. One time trypsinization after R6G treatment is necessary to make the cybrids, but thus far cells have not survived the trypsinization step for cybrid generation. This may be due to the dose of the R6G, confluency of the cells or the cellular characteristics. Currently, we are optimizing the conditions and the dosage of the R6G treatment for different cell types in order to produce cybrids.

Since a drug resistant gene is necessary in the nuclear donor cells for the selection of cybrids after fusion, we have generated puromycin resistant strains of all the cells via transfection. The stable resistant clones with puromycin resistant genes are being used for the dose optimization experiments. Also as a pre-requisite for cybrid clone confirmation, we are also sequencing the DNA from mitochondrial and nuclear donor cells for mitochondrial and nuclear DNA mutations by Next Generation Sequencing (NGS) so that we can confirm the origin of the cybrids after cellular fusion. We feel that sequencing techniques are rapidly the need to use variable number of tandem repeat studies to test nuclear origin.

Key Accomplishments:

1. Isolation of more than 75% of the DNA samples required for the entire project.
2. Validation of a more robust sequencing technique using single amplicon long-range PCR that permits deep coverage (10,000-20,000X on average) of the mitochondrial genome.
3. Initial mapping of the DNA variants to mitochondrial genes.
4. Illumina African Admixture mapping of the initial 75 subjects demonstrating that:
5. Initial steps toward creating the Rho0 cell lines necessary for cybrid generation –
    a. puromycin resistant cell lines have been created
    b. puromcyin resistant cells are being treated with either ethidium bromide or Rhodamine6G to deplete the cells of mitochondria. Conditions for each of these strategies are being optimized.

Reportable Outcomes:

No completed reportable outcomes have been accomplished as yet, but cybrid cell lines may soon be available. We are in the process of creating the Mitochondrial database with mtDNA sequence data, African American Admixture Data and Clinical Data.

Conclusions: Thus far we have made significant progress toward mitochondrial sequencing of the 2000 subject samples required for this project. In concert with Dr. David Wheeler in the Human Genome Sequencing Center and Dr. Lee-Jun Wong, we have implemented a new more rapid and much more powerful technique for sequencing mitochondrial DNA using single amplicon long-range PCR. This technology will permit us to rapidly sequence a large number of samples using NGS technology at a depth of coverage of 10,000-20,000X. This technology allows much more accurate base calls and improves the limit of detection for heteroplasmy from about 15% with Sanger sequencing to ~ 1-2% with the long range primer technique. Three hundred DNA samples are now in the sequencing pipeline with several hundred more to be submitted within the next few weeks. We envision that this technology will permit much more rapid generation of mtDNA sequence with much less DNA and less "contamination" by nuclear DNA. We are hopeful that this technology will be applicable to the FFPE DNA once optimized, but we are continuing to work on the overlapping primers in case they are needed for Sanger sequencing. We have initiated the genotyping for Admixture mapping which required only 25 ng of DNA – much less DNA than we had initially thought would be required. Finally we have made quite a bit of progress in creating the Rho^0 cells which will be required for the cybrid experiments. To date we have created the puromycin resistant cell lines and we are testing two alternative methods for generating cybrids. We are confident that one or both of these techniques will permit us to generate the cell lines we need.

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